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RGD-containing Peptides Inhibit the Synthesis of Myelin-like Membrane by Cultured Oligodendrocytes

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Abstract. A synthetic peptide derived from the fibronectin cell-binding domain, GRGDSP, inhibits the adhesion of rat oligodendrocytes to a number of substrates. However, while GRGDSP inhibited the adhesion of cells in a short term adhesion assay, the presence of the peptide did not prevent cells from adhering and thriving in longer term culture. The morphological characteristics of individual cells cultured with 0.1 mg/ml GRGDSP were similar to untreated cultures; small rounded cell bodies radiating numerous fine processes. Peptide-treated cultures were inhibited in their ability to produce myelin specific components. The characteristic developmental peak in sulfolipid synthesis which occurs both in vivo and in vitro was completely inhibited when cells were cultured with GRGDSP. In addition, the synthesis of myelin basic protein was inhibited. Ultrastructurally, cells treated with GRGDSP showed a greatly reduced number of multilamellar myelin-like membrane figures than cells grown without peptide or those grown with GRADSP. Cultured oligodendrocytes did not become sensitive to inhibition of sulfolipid synthesis by GRGDSP until a period immediately preceding the peak in sulfolipid biosynthesis. The effects of pretreatment with peptide for 5 d before this time were completely reversible. Pretreatment which extended into the time of peak myelin synthesis resulted in permanent impairment in the cell's ability to synthesize sulfolipid. The oligodendrocyte's ability to synthesize a myelin-like membrane in culture is, in part, inherent since it occurs in the absence of neurons. The present results indicate that myelin membrane production is also subject to external control since it appears that occupancy of an RGD-dependent cell surface receptor during a critical period of in vitro development is required for the oligodendrocyte to produce myelin-like membrane.

CELLS in a developing tissue derive information from cell surface contact with both other cells and with components of the extracellular matrix which guides their positional and metabolic fates. The interaction of fibronectin with its cell surface receptors is among the best characterized of such cell surface contacts (for reviews see references 2, 31, and 40). The region within the fibronectin molecule that contains cell-binding activity has been localized to a tripeptide of arginine-glycine-aspartic acid (RGD in single letter code). Synthetic peptides which contain this sequence inhibit fibronectin's interaction with its receptors and, when immobilized, mimic fibronectin's cell-binding activity.

In addition to fibronectin, numerous proteins have been shown to bind to cells in an RGD-dependent manner, including vitronectin, fibrinogen, von Willibrand's factor, thrombospondin, and type I collagen (for reviews see references 32 and 33). Cell surface receptors for many of these extracellular ligands have been purified and characterized (for reviews see references 13 and 28). In most cases the receptors appear to be specific for a single ligand but in at least one case a single receptor complex binds several different ligands. Purified receptors show biochemical similarity both in primary sequence and in subunit composition, suggesting that they are related members of a group of proteins with roles in cell adhesion.

The extracellular matrix has been shown to regulate transcriptional activity in several cultured cell types (5, 18). It has been shown that certain transformed cells lack cell surface fibronectin and that adding fibronectin to these cultures restores an untransformed phenotype (3, 10, 41). The fibronectin receptor of avian cells has been implicated in serving as a link between the cytoskeletal system and the extracellular environment (9, 11, 29). Fibronectin has been shown to have a role in cell migration both in vitro (16, 21) and in vivo (6, 26). However apart from general roles in anchorage, maintenance of cell shape, and cell migration, the cellular functions which are responsive to occupancy of these RGD-dependent receptors remain to be clarified. Recently, evidence has been presented suggesting that occupancy of the RGD-dependent receptor Integrin, previously known as CSAT antigen, is coupled to the differentiation of myoblasts

(24). Immature cells cultured with antibodies to a subunit of Integrin, or with RGD-containing peptides, are prevented from differentiating into myotubes.

Cell surface contacts are of obvious importance in the developing brain. The cellular complexity of the organ precludes the study of many of these interactions. Myelin formation in the immature central nervous system (CNS) is a developmentally regulated event in which the role of cell surface contacts is more readily studied since it involves only two classes of cells. CNS myelin is formed as an extension of the plasma membrane of a single cell type, the oligodendrocyte, which surrounds and ensheathes a subset of neuronal processes. The biogenesis of the myelin membrane is under strict temporal regulation, occurring chiefly in a brief and circumscribed time period during the perinatal development of the brain. Another level of regulation is evident in that not all axons are myelinated; some selection of myelin competent axons must occur. Cell surface contacts which accompany myelination must, at the very least, include a specific recognition event between the oligodendrocyte and axon. It is conceivable, as well, that aspects of the interaction between oligodendrocyte and axon are involved in the temporal control of myelin biosynthesis.

We and others have shown that oligodendrocytes maintained in culture in the absence of neurons synthesize a membrane that is myelin-like by both biochemical and morphological criteria (7, 15, 27, 30, 34, 36). We have been examining the cell surface events which lead to the ability of these cells to produce this myelin-like membrane in culture. Building on the knowledge that oligodendrocytes interact with several extracellular proteins in an RGD-dependent manner (8), we have examined the role that RGD-dependent interactions play in the maturation of oligodendrocytes and in the development of their myelin-like properties.

Materials and Methods

Materials

Polystyrene 24-well plates, both treated and untreated for tissue culture use, were manufactured by Linbro and obtained from Flow Laboratories (McClean, VA). Calf serum was obtained from Hyclone (Logan, UT). $[^{35}S]H_2SO_4$ was purchased from ICN. Antisera to myelin basic protein was the gift of Dr. Scott D. Linthicum, Department of Pathology, University of Texas Medical School (Houston, TX). All other chemicals were from standard commercial suppliers.

Methods

Cell Culture. Purified oligodendrocytes were prepared from neonatal rat cerebral cortex by a modification of the method of McCarthy and de Vellis (22), as described (30). This method is based on the differential adhesion of oligodendrocytes and astrocytes in mixed glial cultures. Oligodendrocytes are released into the culture medium upon extended shaking, while astrocytes remain attached to the plastic surface. Oligodendrocytes released into culture medium were filtered through a 20 μM stainless steel mesh (Cellector, Bellco, Vineland, NJ) and were replated at a density of $\sim 5 \times 10^5$/well in 24-well plates on either tissue culture treated plastic or on astroglial matrix (AGM)[1]. AGM was prepared by water lysis of mixed glial cells (8). After allowing cells 8 h to attach to the surface, as much media was removed as possible without uncovering and disturbing the cell layer. The total volume per well was then adjusted to 1.0 ml with fresh media. Cultures were maintained in DME/F12 supplemented with 5% calf serum. Cells were grown in the presence of various peptide inhibitors by addition of an aliquot of a 10× stock made up in the same media.

1. *Abbreviation used in this paper*: AGM, astroglial matrix.

We routinely date our cultures with the convention of "equivalent brain age." We use this to mean the age of the rats at the time of dissection plus the number of days in culture (30). All references to the age of cells make use of this convention. Oligodendrocytes are isolated as a pure population from mixed glial cells on day 9.

SDS-PAGE/Immunoblotting. Polyacrylamide slab gels were prepared with SDS using the buffer system of Laemmli (17). Samples to be analyzed were prepared in buffer containing 62.5 mM Tris, 3% SDS, 10% glycerol, 5% β-mercaptoethanol, pH 6.8. Samples were heated at 100°C for 5 min and run on 15% gels with a 4.5% stacking layer. Gels were silver stained by the method of Merril (25).

Western blot analysis was carried out by electrophoretic transfer to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) according to the method of Towbin (37). The membranes were blocked in PBS with 2% nonfat milk and 20% methanol. The antibody incubations and color development were carried out as described elsewhere (8).

Metabolic Labeling. Cells grown in 24-well plates on AGM were labeled with 2–5 μCi $[^{35}S]H_2SO_4$ in 0.5 ml per well low sulfate DME/F12 with 1% CS. The labeling time was 16 h unless otherwise noted. When the labeling time was extended beyond 24 h, the low sulfate DME was supplemented with 2% calf serum and the volume per well was increased to 1.0 ml. To harvest cells, the cell layer was washed once with PBS then extracted with 0.4 ml 4 M guanidine HCl, 50 mM EDTA, 50 mM sodium acetate, 0.1 M aminocaproic acid, 1.0% CHAPS, pH 5.8. This extract was sonicated to disperse the cellular material. An aliquot of this extract was removed for protein determination and the remainder was extracted for lipid analysis with 10 vol of isopropanol:hexane (2:3). The lipid extracts were vortexed and the resulting two phase system was clarified by centrifugation. A portion of the upper phase was transferred to scintillation vials to measure $[^{35}S]$sulfolipid. Analysis of the lipid extract by TLC on silica G plates revealed that 95% of the total radioactivity co-migrated with a sulfatide standard.

Synthetic Peptides. Peptides were prepared by Dr. Janice Young of the UCLA Peptide Synthesis Facility in the Department of Biological Chemistry. The peptides were synthesized on an Applied Biosystems, Inc. (Foster City, CA) 430A Peptide Synthesizer using version 1.3 software. The peptides were cleaved from the resin with anhydrous hydrogen fluoride with 10% anisole. The cleaved peptides were extracted with ether, dissolved in 1% acetic acid and lyophilized. Peptides were either used without further purification or were additionally purified by two cycles of gel filtration on Sephadex G10 (Pharmacia, Upsalla, Sweden) in H_2O, followed by lyophilization and a wash with cold ether. These additional purification steps did not alter the activity in either adhesion or long term culture experiments.

Electron Microscopy. Cell cultures grown in 24-well plates on plastic coverslips (Thermanox, Lux) were washed twice in PBS. The cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate, pH 7.2, for 48 h at 4°C. Cells were postfixed in 1% OsO_4 in the same buffer, dehydrated, and spurr embedded. The sections were examined on a JEOL 100-EX electron microscope.

Myelin Isolation. Myelin was isolated from cultured oligodendrocytes and from whole brain by a procedure originally developed for whole brain (1). Oligodendrocytes were grown in 75 cm^2 flasks, harvested at 24 d "equivalent brain age" by scraping into 0.88 M sucrose, and homogenized in a tight fitting Dounce homogenizer. The final volume was 2.0 ml per flask. These homogenates were transferred to centrifuge tubes and each tube was overlaid with 2.0 ml 0.34 M sucrose. These two step gradients were centrifuged at 75,000 *g* for 45 min in a SW 60 rotor. The material accumulated at the interface was collected and diluted with 10 vol of cold distilled water. The collected membranes were Dounce homogenized and pelleted at 20,000 *g* for 20 min. This osmotic shock procedure was repeated and the final pellet was saved as the crude myelin fraction. Myelin from whole brain was prepared at the same time from 24-d-old littermates of those animals used to prepare the primary cultures. The percentage of protein recovered in the myelin-like fractions relative to that in the cell homogenates was used to quantitate membrane production under the various conditions.

Protein Determination. Protein values were estimated by the bicinchoninic acid method using a kit supplied by Pierce (Rockford, IL) with BSA as standard (35).

Results

Inhibition of Sulfolipid Synthesis by GRGDSP

Oligodendrocytes adhere to a number of different substrates in an RGD-dependent manner. However, these cells can be

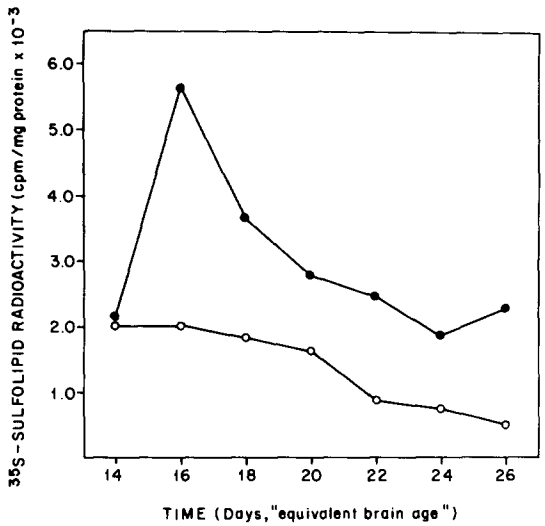

Figure 1. Inhibition of developmental pattern of [^{35}S]sulfolipid synthesis by GRGDSP. Oligodendrocytes obtained from mixed glial cells were plated on AGM. The cultures were supplemented with 0.1 ml of 1.0 mg/ml GRGDSP in DME/F12 with 5% calf serum (○) or with 0.1 ml DME/F12 with 5% calf serum (●). The cultures were fed every other day by replacing one half the total volume of each well with either culture medium alone or medium supplemented with GRGDSP, maintaining the initial concentration of peptide. Cultures were labeled for 16 h in low sulfate DME containing 1% calf serum and 4.0 μCi [^{35}S]H_2SO_4/ml beginning on the day shown. The cell layer was washed, solubilized and lipid extracted as described in Materials and Methods. Each bar represents the average of 3 separate determinations and the variation between samples was less than 10%.

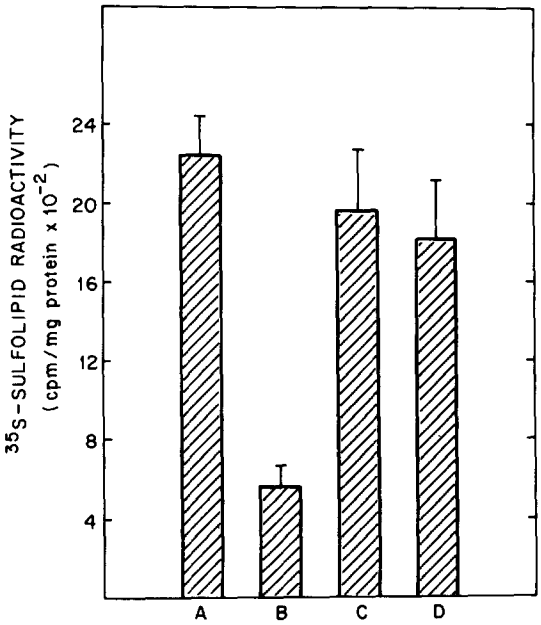

Figure 2. Specificity of peptide inhibition of [^{35}S]sulfolipid synthesis. Oligodendrocytes plated on AGM were cultured with no peptide additions until day 15, at which time they were supplemented with the appropriate peptide solution. Cells were grown in media alone (*A*), or in media with 0.1 mg/ml GRGDSP (*B*), 0.1 mg/ml GRADSP (*C*), or 0.1 mg/ml VYPNGA (*D*). The cells were kept in the presence of peptide for three days until day 18 with no further media change. On day 18 the cells were labeled for 16 h in the absence of peptide, harvested, and extracted as described in Materials and Methods. Each bar represents the average of 3 wells and the error bars indicate SD.

maintained in culture in the presence of a concentration of GRGDSP which does not cause a significant amount of cell loss. Cells grown with 0.1 mg/ml GRGDSP show only slight differences in overall morphology. Notable among these changes is a reduced tendency of cells to aggregate into the multicellular clusters which have often been observed with oligodendrocytes in culture (39). Despite no gross differences in individual cell morphology, peptide-treated cultures did show marked differences in their ability to produce myelin specific components. As we have previously shown, pure oligodendrocytes maintained in culture progress through a characteristic developmental change in the ability to produce the myelin-enriched galactolipids sulfatide and cerebroside (30). In addition to these galactolipids, these cells synthesize the major myelin proteins, myelin basic protein and proteolipid protein as well as several minor myelin protein constituents. Moreover, these cultures assemble these myelin components, in the absence of neurons, into a loosely compacted, multilamellar myelin-like membrane. The peak synthetic levels determined in culture when corrected for "equivalent brain age" are similar to those determined in vivo (4, 23). In the experiment shown in Fig. 1, the biosynthetic peak for [^{35}S]sulfolipid in untreated cultures occurred on day 16. When cells were grown in the presence 0.1 mg/ml GRGDSP from the time of plating as pure oligodendrocytes and labeled after various times in culture, the normal developmental pattern of sulfatide synthesis was completely blocked (Fig. 1). Sulfolipid synthetic levels for peptide-treated cultures never exceeded the baseline level of the untreated controls. We have expressed values of [^{35}S]sulfolipid synthesis per unit protein as an approximation of cell number.

As shown in Fig. 2, this inhibition of sulfolipid synthesis is specific to GRGDSP. Two other peptides were examined for their ability to inhibit sulfolipid synthesis. One of these peptides was a hexapeptide with a single substitution of alanine for glycine within the RGD region. The other peptide was an unrelated hexapeptide. These peptides caused little or no inhibition of sulfolipid synthesis.

Timing of GRGDSP Effects

To determine the length of exposure to GRGDSP required for maximal inhibition of sulfolipid synthesis, cultures of pure oligodendrocytes were treated for up to 7 d in media containing 0.1 mg/ml of the peptide. Cells were labeled with [^{35}S]H_2SO_4 on day 17 for 16 h in media that did not contain the peptide. As shown in Fig. 3, sulfolipid synthesis was reduced to 7–15% of control values with 3–5 d of pretreatment, although even a 1-d pretreatment had a significant inhibitory effect.

Oligodendrocytes were viable in culture for several months and continued to synthesize the myelin-enriched galactolipids throughout this time. The cells were variably inhibited by peptide treatment depending on the age of the cultures. While measurable levels of sulfolipid were produced in newly plated cells, this basal level of synthesis did not appear

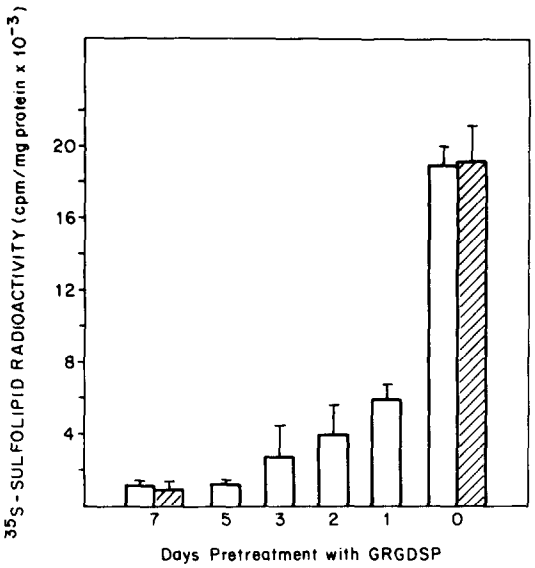

Figure 3. Effects of pretreatment of oligodendrocytes with GRGDSP on [^{35}S]sulfolipid synthesis. Oligodendrocytes were plated on AGM (*open bars*) or on plastic (*hatched bars*) and allowed 8 h to adhere before media was partially removed and replaced with fresh media. Peptides solutions were added to the cultures from 1 to 7 d before labeling, which was carried out on day 18. The values shown for 0 days pretreatment were obtained from cultures which contained no added peptide. In all cases, the cells were labeled for 16 h, harvested, and extracted as described in Materials and Methods. Each bar represents the average of 3 wells and the error bars indicate SD.

to be inhibited by GRGDSP. Inhibition was not apparent until days 14–16. While cells were impaired by peptide treatment at any age in culture after this time, the extent of inhibition was greatest when the cells were labeled during the developmental peak in sulfolipid synthesis (data not shown).

While we have shown that the oligodendrocytes adhered in an RGD-dependent manner to AGM and not to plastic (8), cells plated on either plastic or AGM showed a decrease in sulfolipid synthesis when cultured in the presence of the RGD-containing hexapeptide (Fig. 3). This result might suggest that it is not the interaction of oligodendrocytes with a component of the AGM which is inhibited by culture with peptide, but perhaps, the interaction of oligodendrocytes with each other (see Discussion).

In all of the above labeling experiments, cells were pretreated with peptide and then labeled for 16 h in media that did not contain peptide. To further measure the rapidity of the sulfolipid inhibition, cells were cultured without peptide until the time that lipid synthesis was maximal. The cells were then labeled in media supplemented with 0.1 mg/ml GRGDSP. Cultures were labeled for up to 24 h and compared with control cultures which contained no added peptide. Inhibition of sulfolipid synthesis was evident after only 2–4 h of exposure to peptide (Fig. 4).

Reversibility of Inhibition of Sulfolipid Synthesis

As shown in Fig. 5, the inhibition of [^{35}S]sulfolipid synthesis was reversible. Oligodendrocytes were cultured with and without added GRGDSP from the time of plating on day 9. Peptide treated cultures were then handled in three different ways: (*a*) cells were kept in the presence of GRGDSP for the duration of the experiment (including the labeling period); (*b*) peptide was removed on day 14 for up to 6 d; or (*c*) peptide was removed on day 16 and cultured for an additional 4 d.

The synthesis of [^{35}S]sulfolipid in cells grown in the presence of peptide was depressed relative to controls by 50% in the day 14–16 labeling period, by 70% in days 16–18, and by 69% in days 18–20. When the peptide was removed on day 14, the synthesis of [^{35}S]sulfolipid was essentially at the level of untreated controls. When the peptide was removed from cultures on day 16, the synthesis of sulfolipid remained depressed to the level of cultures continually treated with peptide. Since a maximal inhibition of [^{35}S]sulfolipid synthesis was seen after only 3–5 d of treatment with peptide, it appears that this finding is not simply the result of longer exposure to the peptide, but of exposure at some critical period of the cells in vitro development. Cells treated in such a way never returned to control levels of synthesis even if allowed up to 10 d to recover. Further, if the peptide was removed at any point after day 16, the oligodendrocyte was also permanently impaired in its ability to synthesize sulfolipid in vitro.

Inhibition of Myelin-like Membrane and Myelin Basic Protein Synthesis by GRGDSP

It is conceivable that the effect of the GRGDSP peptide on sulfolipid synthesis is due to a direct inhibition of the enzymes of sulfatide biosynthesis or on altered sulfate uptake

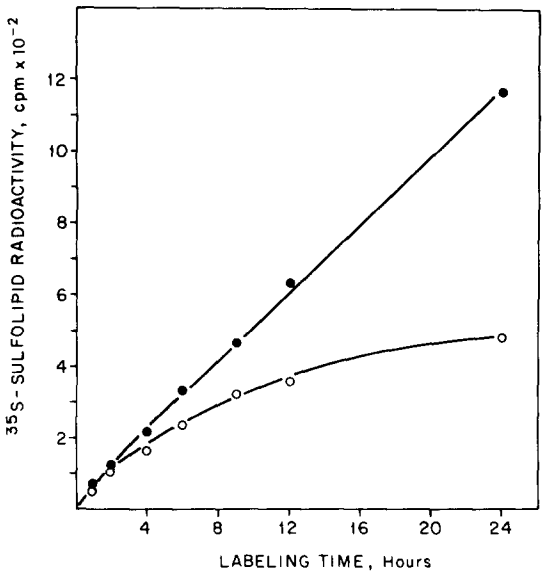

Figure 4. Time course of inhibition of [^{35}S]sulfolipid synthesis by GRGDSP. Oligodendrocytes were cultured for 1 wk until day 16, in the absence of GRGDSP. The cultures were labeled starting on day 16 for 1 to 24 h in media that contained no peptide (●) or in media that contained 0.1 mg/ml GRGDSP (○). [^{35}S]Sulfolipid synthesis was determined as described in Materials and Methods. Each bar represents the average of 3 separate determinations and the variation between samples was less than 10%. The [^{35}S]sulfolipid values were not expressed relative to protein content since the treatment caused no noticeable cell loss and the total protein content of each well did not change over the course of the experiment.

or metabolism, and not a specific effect related to the cells ability to produce a myelin-like membrane in culture. We therefore examined more directly the production of myelin-like membranes in cultures treated with GRGDSP. Cultured oligodendrocytes were grown either in media supplemented with 0.1 mg/ml GRGDSP from day 14 to day 18, in media supplemented with 0.1 mg/ml GRADSP from day 14 to day 18, or without added peptide. All cultures were maintained in media without peptide from day 18 to day 24 at which time they were harvested and used to prepare a crude myelin-like membrane fraction. There is a small degree of cell loss in GRGDSP-treated cultures which was minimized by limiting the length of exposure to peptide to only 4 d. In the experiment shown in Fig. 6, GRGDSP-treated cultures contained 94% of the total protein content of untreated controls. However, when corrected for differences in the amount of protein in the starting homogenate, the protein content of the myelin-like membrane fraction isolated from GRGDSP-treated cultures was 27% of that derived from untreated cultures. For GRADSP-treated cultures this value was 75%.

The content of myelin basic protein in the myelin-like membrane fractions of these cultures is shown in Fig. 6, lane *c*. There is a marked reduction in myelin basic protein content in GRGDSP-treated cultures relative to either GRADSP-treated or untreated cultures. The content of myelin basic protein in the total cellular homogenates of the cultured oligodendrocytes was decreased to the same extent in GRGDSP-treated cultures relative to control cultures (data not shown), suggesting that the peptide's effect is not simply to alter the buoyant density properties of the myelin-like membrane formed.

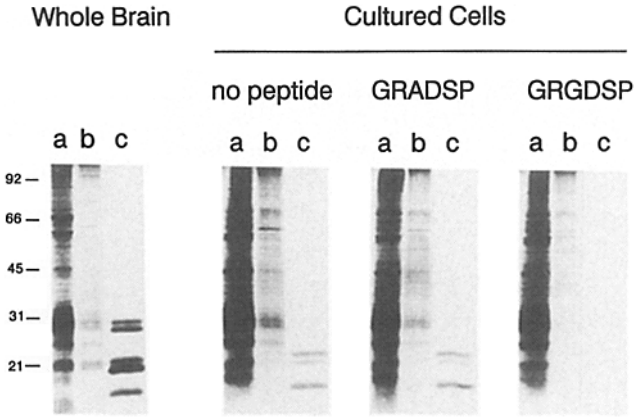

Figure 6. Inhibition of myelin-like membrane production and of myelin basic protein synthesis by GRGDSP. Oligodendrocytes were maintained in 75 cm^2 flasks from day 9 until day 24. Cultures treated with peptide were kept in the presence of 0.1 mg/ml of either GRGDSP or GRADSP from days 14 to 18. The cells were harvested and used for myelin isolation as described in Materials and Methods. Whole brain from littermates of the animals used in the primary dissection was treated in the same way. Silver-stained samples of the total homogenate (lane *a*), of the crude myelin-like fraction (lane *b*), and western blots of crude myelin-like fractions probed with antibodies to myelin basic proteins (lane *c*) are shown. For the samples isolated from cultured cells, 2.5 μg of total protein was applied to lane *a*. The myelin-like membrane fractions isolated for each treatment were suspended in a constant volume relative to the amount of protein in the starting homogenate. The same percentage of these samples (not the same amount of protein) was run in lanes *b* and *c*.

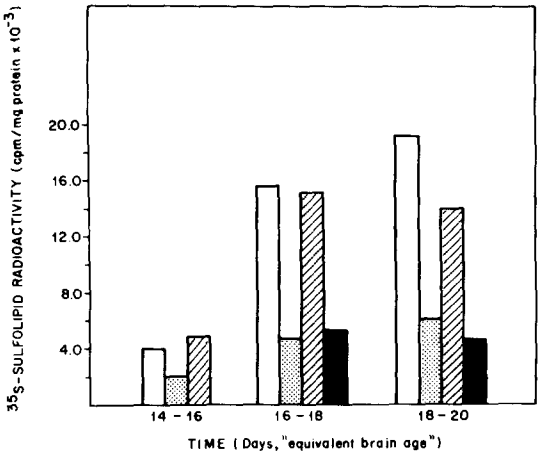

Figure 5. Reversibility of GRGDSP inhibition of [^{35}S]sulfolipid synthesis. Oligodendrocyte cultures were plated on AGM and were maintained in media without added peptide (*open bars*), with 0.1 mg/ml GRGDSP for the duration of the experiment, days 9 to 20, (*stippled bars*), with 0.1 mg/ml GRGDSP from days 9 to 14 and then in media that did not contain peptide (*hatched bars*), or with 0.1 mg/ml GRGDSP from days 9 to 16 and then in media that did not contain peptide (*solid bars*). The cells were labeled for 48 h in low sulfate DME with 2% calf serum in a total volume of 1.0 ml. For cultures which were kept in the presence of peptide throughout, the labeling media was supplemented with peptide to a final concentration of 0.1 mg/ml. Each bar represents the average of 3 separate determinations and the variation between samples was less than 10%.

Cultured oligodendrocytes produce a multilamellar myelin-like membrane in the absence of neurons, that can be readily detected by using electron microscopy. In cultures grown with the control peptide (GRADSP) or without peptide these multilamellar membranes are evident in the proximity of nearly every cell examined. As shown in Fig. 7 *A*, in cultures treated with GRADSP for 4 d between days 14 and 18, these membranes were very prominent and commonly displayed as many as 10 semi-compacted lamellae. In contrast, in cultures treated with GRGDSP for this same length of time, multilamellar membrane was very infrequently observed. Less than 20% of the treated oligodendrocytes displayed any multilamellar membrane in their extracellular surroundings and, when it was observed, the lamellae never exceeded 2–3 layers. The micrograph shown in Fig. 7 *B* represents an oligodendrocyte from the minor population of cells that had extracellular figures. Note the reduced amount and the reduced complexity of the membranous elements.

Discussion

We are interested in the cell surface adhesion and signaling events which precede and accompany myelination of neuronal axons by oligodendrocytes. Recently, in a system in which oligodendrocytes isolated from adult ovine brain were cultured either in suspension or attached to a polylysine-treated surface, nonspecific adhesion was shown to influence the functional state of the cells. The synthesis of the major myelin proteins, proteolipid protein and myelin basic pro-

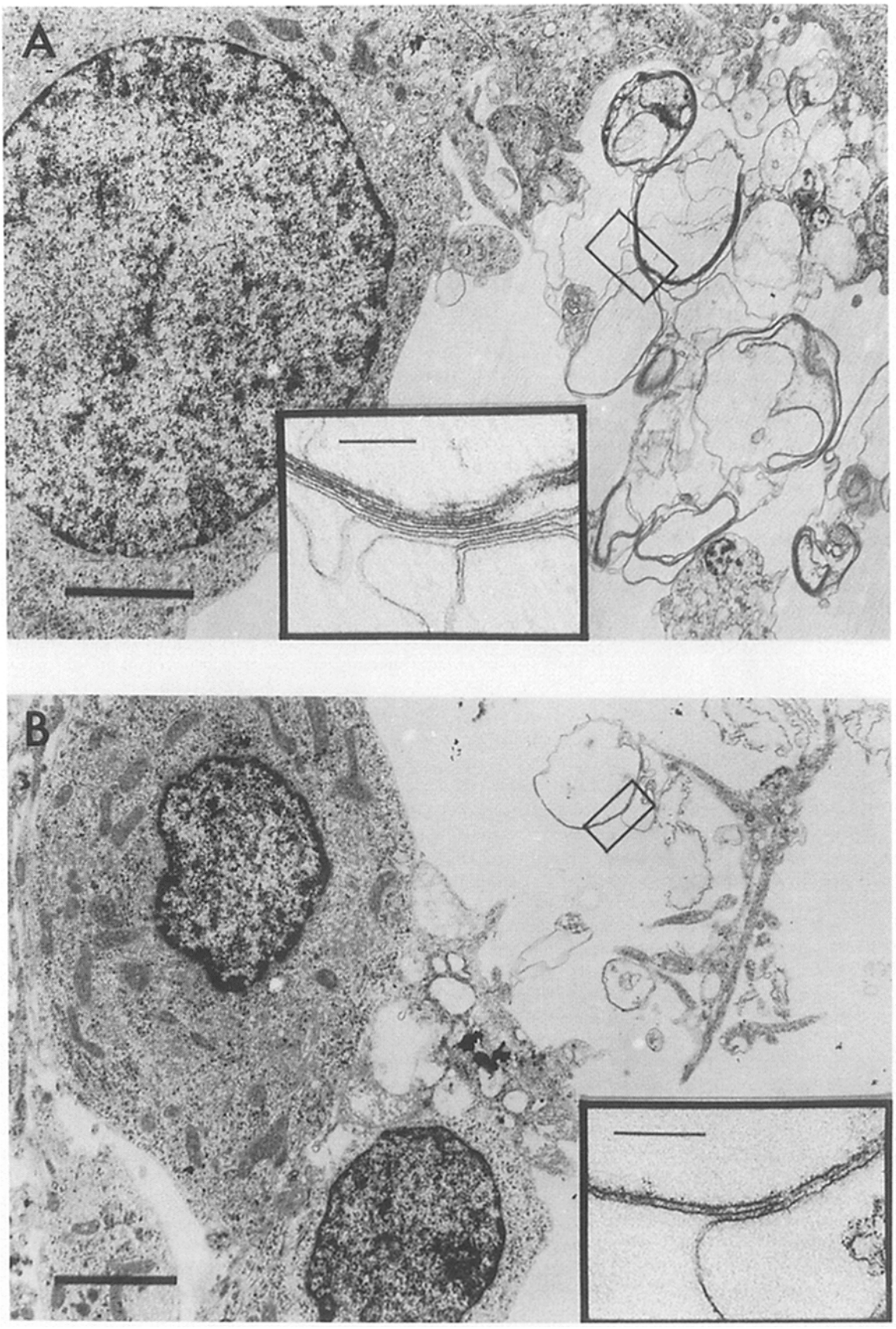

Figure 7. Ultrastructure of peptide-treated oligodendrocytes. Oligodendrocytes were grown on coverslips in 24-well plates. Cells were kept in the presence of 0.1 mg/ml GRADSP (*A*) or GRGDSP (*B*) from days 14 to 18. The cells were fixed on day 24 and processed as described in Materials and Methods. The field shown in *A* is typical of cultures without added peptide or treated with GRADSP (i.e., nearly every cell is found to have numerous myelin-like figures in the surrounding extracellular space). The field in *B* shows an example of the minor population of cells which have surrounding myelin-like figures. Bars: (*A* and *B*) 1.5 μm; (*insets*) 250 nM.

tein, was enhanced and the phosphorylation of myelin basic protein was increased in adherent cells (38, 42). Oligodendrocytes are capable of forming adhesive associations with substrate by both RGD-dependent and independent (e.g., via charge interactions with polyion-coated surfaces) mechanisms (8). By culturing cells with a level of GRGDSP which does not cause significant cell detachment, we have been able to specifically perturb the RGD-dependent contacts and to examine the contributions of these interactions to the development of myelin-like properties in culture.

We have made extensive use of sulfolipid as a marker for myelination. In cultures of neonatal rat oligodendrocytes, >95% of the total [^{35}S]sulfolipid synthesized is sulfatide, a sulfated galactolipid with a very limited tissue distribution (12). In the brain, sulfatide is essentially restricted to the myelin membrane and is widely used as a marker for both myelin and for the oligodendrocyte. We have used the measurement of sulfolipid as a convenient and accurate estimate of sulfatide synthesis and of myelin-like membrane synthesis.

The effects of the GRGDSP peptide on long term cultures of oligodendrocytes were not due to cytotoxicity. Control peptides synthesized under the same protocol had little or no effect on the parameters measured. Extensive purification of the active peptide by gel filtration and ether extraction caused little or no decrease in its inhibitory activity. Furthermore, cells cultured in the presence of peptide had typical oligodendrocyte morphology and remained viable for the duration of our observation, up to day 28.

Oligodendrocytes produced measurable amounts of sulfolipid for as long as they were maintained in pure culture (in our experiments pure oligodendrocytes were isolated from mixed glial cultures on day 9). Superimposed on this basal level of synthesis is a biosynthetic peak, the timing of which immediately precedes the appearance of myelin figures in the cultures. GRGDSP completely inhibited the characteristic biosynthetic peak observed with these cells in culture, though the cells continued to synthesize sulfolipid at a basal level (Fig. 1).

The synthesis of sulfolipid was not inhibited by GRGDSP until the time that sulfolipid synthesis began to peak on days 14–16. After cells became sensitive to inhibition on days 14–16, they remained so, although they were most susceptible during the time that lipid synthesis was maximal. Moreover, treatment with peptide was completely reversible if the peptide was removed on or before day 14. Treatment for 5 d from day 9 to 14 did not appear to effect the subsequent development of myelin properties, causing neither a diminution nor a delay in the observed biosynthetic peak. Cultures treated after day 14 with peptide were permanently impaired in their ability to synthesize sulfolipid.

The effects of the GRGDSP peptide were not confined to sulfolipid synthesis, we also observed an overall reduction in production of myelin-like membrane, assayed both biochemically and morphologically. Myelin-like membrane isolated from cultured cells was similar to whole brain when compared on SDS–PAGE, although the membrane from cultured cells contained many minor bands not found in samples from whole brain and which did not seem to be effected by peptide treatment. Both membrane isolated from whole brain and from cultured cells was enriched in the myelin specific proteins MBP and proteolipid protein. The GRGDSP peptide caused a substantial reduction both in the amount of overall membrane proteins produced and in the amount of MBP detected by western blotting. The morphological differences were even more dramatic; peptide-treated oligodendrocytes produced far fewer multilamellar membrane figures than control cultures and those membranes were both less complex and less compacted.

The results summarized above suggest that the biochemical changes responsible for the production of myelin-like membrane appear to be dependent on occupancy of the cell's RGD-dependent receptor during a critical period of in vitro development. This critical period occurs between days 14 and 16 and immediately precedes the time of peak lipid synthesis (8). When cultures were treated with GRGDSP from days 14 to 18, then cultured without peptide until day 24, they were inhibited in the production of myelin-like membranes, even though the time of peptide treatment preceded the time when myelin-figures became prominent in the cultures. In a culture system in which oligodendrocytes are maintained in a chemically defined medium, the cells show the characteristic peak in sulfolipid synthesis that we and others have observed, only when cultured with thyroxine (14). It is interesting to note that there also appears to be a critical period which immediately precedes the time of peak synthesis when thyroxine is absolutely required.

GRGDSP inhibited the adhesion of oligodendrocytes to AGM, but had no effect on binding to polystyrene, which was not modified for tissue culture use. However, given sufficient time, oligodendrocytes adhered to unmodified polystyrene and developed the same myelinogenic properties as cells grown on AGM. The observation that cells plated on plastic were inhibited in sulfolipid synthesis by the addition of GRGDSP and the fact that peptide treatment seemed to effect the aggregation of cells might suggest that the interaction disrupted by peptide treatment was not between cell and substrate, but rather between cells. Oligodendrocytes have been shown to interact extensively with other oligodendrocytes via tight junctions both in vivo (19) and in culture (20). The tendency of oligodendrocytes to cluster has been noted by others working with a similar culture system (39). It is conceivable that these intercellular associations are functionally important to the oligodendrocyte and that they are mediated by RGD-dependent interactions. While the bulk of the information on RGD interactions has been derived from studies on cellular interactions with extracellular matrix constituents, there is also evidence that these interactions are important in specific cell–cell associations. A group of leukocyte cell surface proteins, the LFA-1, Mac-1, and p150,95 family, which shows extensive homology to each other as well as to the receptors for fibronectin and vitronectin, has been shown to be involved in antigen-independent binding between leukocytes as well as leukocyte binding to capillary endothelial cells (13).

Another explanation for the observation of inhibition of sulfolipid synthesis in cells on plastic as well as AGM is that, while AGM is the product of cultures which contain both oligodendrocytes and astrocytes, oligodendrocytes themselves may synthesize the protein component of AGM which binds cells in an RGD-dependent manner. However, when pure cultures of oligodendrocytes were grown for 7 d and then used to prepare a matrix by the same water lysis procedure used to make AGM, the surface did not promote adhesion above the level of serum-treated plastic (Cardwell,

M. C., and L. H. Rome, unpublished observation). While the component of AGM does not appear to be derived from serum, serum-treated plastic does exhibit ~16% of the RGD-dependent binding of AGM. Perhaps this level of binding is sufficient for the cells to develop their myelinogenic properties. If serum provides a soluble ligand for the cell surface component of the oligodendrocyte, one would expect cells grown on either plastic or AGM to be equally effected by peptide treatment.

We have demonstrated the importance of RGD-dependent contacts in the development of myelin properties in culture. More detailed analysis of the role that RGD interactions play in myelination awaits purification of the oligodendrocyte receptor. Questions of immediate interest include the localization of the receptor on the cell surface. Does it for example localize to the tips of the growing processes which one might expect to be the first part of the oligodendrocyte to make contact with a receptive axon. What are the changes which occur in the cell during day 9 to day 16 which make it susceptible to inhibition by peptide? Since the adhesion of newly isolated oligodendrocytes (day 9) is inhibited by GRGDSP, the receptor is present on the cell surface at a time before the cells become sensitive to the inhibition of sulfolipid synthesis. The changes which confer this sensitivity may involve an increase in receptor number and/or the functional association of the receptor with an effector system responsible for linking cell surface adhesion/signaling events to the production of myelin membrane components.

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